NRH-quinone oxidoreductase 2 (NQO2) (Long and Jaiswal 2000). Quinones are highly reactive molecules and readily generate unstable semiquinones by reduction. Semiquinones undergo redox cycling in the presence of molecular oxygen and this leads to formation of highly reactive oxygen species (ROS). ROS and semiquinones cause oxidative stress. NQOs catalyze two-electron reduction competing with the one-electron reduction to detoxify quinones and their derivatives, and protect cells against damage associated with redox cycling and oxidative stress (Long and Jaiswal 2000; Ross et al. 2000). The insertion / deletion (I/D) polymorphism in the promoter region of the NQO2 gene has shown positive correlation with idiopathic Parkinson's disease (Harada et al. 2001), schizophrenia (Harada et al. 2003) and alcohol withdrawal symptoms (Okubo et al. 2003), although no difference was detected for the polymorphisms of the NQO1 gene and the other polymorphic loci of the NQO2 gene. Based on the role of oxidative stress in MAP-induced neurochemical changes, it is of great interest to examine association between polymorphisms of the NQO genes and MAP abusers. The purpose of the present study was to elucidate genetic vulnerability in Japanese MAP abusers associated with the polymorphisms of the NQO genes. In this study, we examined the association between the polymorphisms in the NQO1 gene (Pro187Ser) and the promoter region of the NQO2 gene and MAP abusers in Japan.

Materials and methods

The research was performed after obtaining approval from the ethics committees of each institute of Japanese Genetics Initiative for Drug Abuse (JGIDA), and all subjects provided written informed consent to the use of their DNA samples for this research. The subjects were 191 patients with MAP dependence and psychotic disorder meeting International Classification of Diseases version 10 (ICD-10)– Diagnostic Criteria for Research (DCR) criteria (F15.2 and F15.5) who were out-patients or in-patients of psychiatric hospitals of JGIDA, and 207 age-, gender- and geographical origin-matched normal controls were recruited mainly from medical staffs that had no past and family history of drug dependence or psychotic disorders (Table 1). All subjects were Japanese. Genomic DNA was extracted from whole blood obtained from the subjects. The patients were classified into three groups by some clinical features: (1) patients with MAP psychosis; (2) patients experienced or not experienced spontaneous relapse; and (3) patients with or without polysubstance abuse. Patients with MAP psychosis were also divided into two subgroups according to the duration of psychotic state: patients with prolonged-type MAP psychosis, whose psychotic state continued for more than 1 month; and patients with transient-type MAP psychosis, whose psychotic state improved within 1 month.

The polymorphism of a C to T substitution in exon 3 of the NQO1 gene (Pro187Ser) was identified by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) using *Hinf* I (New England Biolabs Inc., Beverly, MA, USA). PCR primers were as follows: forward primer, 5′-TCCTCAGAGTGGCATTCTGC-3′ and reverse primer, 5′-TTCTCCTCATCCTGTACCTCT-3′; the annealing temperature was 58°C. The PCR product [240 base pairs (bp)] were cut by the restriction enzyme of *Hinf* I, producing two fragments of 215 and 25 bp for the C/C genotype, four fragments of 215, 180, 35 and 25 bp for the C/T genotype and two fragments of 180 and 35 bp for the T/T genotype. To genotype the polymorphism of I/D of 29 bp nucleotides in the promoter region of the NQO2 gene, PCR analysis was performed using the primers 5′-CTGCCTGGAAGTCAGCAGGGTC-3′ and 5′-CTCTTTACGCAGCGCGCCTAC-3′; the annealing temperature was 64°C. The PCR product size for homozygous genotype of insertion (I/I) was 291 bp and that for homozygous genotype of deletion (D/D) was 262 bp. Heterozygous genotype of insertion and deletion (I/D) made 310-bp heteroduplex DNA fragment in addition to 291-bp and 262-bp PCR products (Harada et al. 2001).

Differences in the distribution of genotypes between patients and controls were analysed using Fisher's exact probability test based on a two-sided p value. The comparison of mean age between the two groups was

Table 1. *Characteristics of the study population*

Characteristics	Controls	Abusers	p value
Number of subjects (male/female)	207 (161/46)	191 (151/40)	0.808*
Mean age, years (SD)	36.5 (11.9)	36.7 (10.6)	0.852†
MAP psychosis		147	
Transient		81	
Prolonged		60	
Unknown		6	
Spontaneous relapse			
Positive		61	
Negative		106	
Unknown		24	
Polysubstance abuse			
Yes		113	
No		52	
Unknown		26	

*Comparison of the male/female ratio between the two groups was performed by a Fisher's exact probability test. †Comparison of mean age between the two groups was performed by an unpaired t-test.

performed by an unpaired *t*-test (SPSS J for Windows 12.0.1 J, SPSS Japan Inc., Tokyo). Significance for the results was set at $p < 0.05$.

Results

The genotype distributions of NQO1 gene and NQO2 gene in patients and controls were in good agreement with the values expected from Hardy–Weinberg equilibrium. The genotype and allele frequencies for the polymorphism (Pro187Ser) of the NQO1 gene did not differ significantly between each subgroup of patients and controls (Table 2). The genotype and allele frequencies for I/D polymorphism in the promoter region of the NQO2 gene are summarized in Table 3. There was a significant difference between patients with prolonged-type MAP psychosis and controls ($p = 0.038$). In addition, there was a tendency to different genotype distribution of the NQO2 gene polymorphism in patients with MAP psychosis compared to controls ($p = 0.085$). No difference was detected in the genotype distribution between patients in the other subgroups and controls. The allele frequencies for I/D polymorphism in the promoter region of the NQO2 gene were not different between each subgroup of patients and controls. There was no gender difference between patients and controls (Table 1).

Discussion

We found that the I/D polymorphism in the promoter region of the NQO2 gene was associated with the prolonged type of MAP psychosis. It has been shown that the I/D polymorphism in the promoter region of the NQO2 gene was associated with genetic vulnerability to idiopathic Parkinson's disease (Harada et al. 2001) and schizophrenia (Harada et al. 2003). Furthermore, it has been reported that the I/D polymorphism in the promoter region of the NQO2 gene plays a role in the pathogenesis of alcoholism and alcohol withdrawal symptoms (Okubo et al. 2003). Therefore, our finding is of interest as MAP psychosis is similar to positive symptoms of schizophrenia (Sato et al. 1992).

Table 2. *Genotype and allele frequencies for the polymorphism (Pro187Ser) of the NQO1 gene*

		Genotype (%)				Allele (%)		
	n	CC	CT	TT	*p* value	C	T	*p* value (OR, 95% CI)
Controls	207	85 (41.1)	95 (45.9)	27 (13.0)		265 (64.0)	149 (36.0)	
Patients	191	69 (36.1)	97 (50.8)	25 (13.1)	0.577	235 (61.5)	147 (38.5)	0.510 (1.11, 0.83–1.48)
MAP psychosis	147	55 (37.4)	75 (51.0)	17 (11.6)	0.642	185 (62.9)	109 (37.1)	0.812 (1.05, 0.77–1.43)
Transient	81	36 (44.4)	35 (43.2)	10 (12.3)	0.886	107 (66.0)	55 (34.0)	0.645 (0.91, 0.62–1.34)
Prolonged	60	17 (28.3)	36 (60.0)	7 (11.7)	0.139	70 (58.9)	50 (41.1)	0.284 (1.27, 0.84–1.92)
Spontaneous relapse								
Positive	61	24 (39.3)	28 (45.9)	9 (14.8)	0.939	76 (62.3)	46 (37.7)	0.749 (1.08, 0.71–1.63)
Negative	106	34 (32.1)	60 (56.6)	12 (11.3)	0.206	128 (60.4)	84 (39.6)	0.383 (1.17, 0.83–1.64)
Polysubstance abuse								
Yes	113	41 (36.3)	53 (46.9)	19 (16.8)	0.556	135 (59.7)	91 (40.3)	0.306 (1.20, 0.86–1.67)
No	52	17 (32.7)	31 (59.6)	4 (7.7)	0.221	65 (62.5)	39 (37.5)	0.820 (1.07, 0.68–1.66)

Statistical analysis was performed by a Fisher's exact probability test (vs. control). OR, odds ratio; 95% CI, 95% confidence interval.

Table 3. *Genotype and allele frequencies for the insertion / deletion (I / D) polymorphism in the promoter region of the NQO2 gene*

		Genotype (%)				Allele (%)		
	N	I / I	I / D	D / D	*p* value	I	D	*p* value (OR, 95% CI)
Controls	207	123 (59.4)	74 (35.7)	10 (4.8)		320 (77.3)	94 (22.7)	
Patients	191	117 (61.3)	59 (30.9)	15 (7.9)	0.336	293 (76.7)	89 (23.3)	0.866 (1.13, 0.74–1.44)
MAP psychosis	147	93 (63.3)	40 (27.2)	14 (9.5)	0.085	226 (76.9)	68 (23.1)	0.928 (1.02, 0.72–1.46)
Transient	81	48 (59.3)	27 (33.3)	6 (7.4)	0.655	123 (75.9)	39 (24.1)	0.742 (1.08, 0.70–1.65)
Prolonged	60	40 (66.7)	13 (21.7)	7 (11.7)	0.038*	93 (77.5)	27 (22.5)	1.000 (0.99, 0.61–1.61)
Spontaneous relapse								
Positive	61	39 (63.9)	18 (29.5)	4 (6.6)	0.578	96 (78.7)	26 (21.3)	0.805 (0.92, 0.56–1.51)
Negative	106	64 (60.4)	32 (30.2)	10 (9.4)	0.229	160 (75.5)	52 (24.5)	0.619 (1.11, 0.75–1.63)
Polysubstance abuse								
Yes	113	68 (60.2)	36 (31.9)	9 (8.0)	0.477	172 (76.1)	54 (23.9)	0.769 (1.07, 0.73–1.57)
No	52	35 (67.3)	12 (23.1)	5 (9.6)	0.128	82 (78.8)	22 (21.2)	0.793 (0.91, 0.54–1.54)

Statistical analysis was performed by a Fisher's exact probability test (vs. control). *$p < 0.05$ vs. controls. OR, odds ratio; 95% CI, 95% confidence interval.

It has been reported that the expression of NQO2 in the D/D genotype of the NQO2 gene is lower than that in the I/I genotype (Harada et al. 2003), suggesting that insufficient NQO2 upregulation and/or activity might be vulnerable to Parkinson's disease (Harada et al. 2001) and schizophrenia (Harada et al. 2003). MAP is also known to cause oxidative stress and neurotoxicity, leading to the irreversible damages to the brain. Insufficient NQO2 activity could not provide enough neural protection against a large amount of DA-quinones derived from DA released by MAP. In this study, the percentage (11.7%) of D/D of the NQO2 gene in patients with prolonged-type MAP psychosis was approximately twofold higher than that (4.8%) of controls (Table 3). Therefore, it is likely that lower expression of NQO2 may contribute to the prolongation of MAP psychosis.

The homozygous T genotype of the NQO1 genes is a risk factor for breast cancer (Menzel et al. 2004), lung cancer (Xu et al. 2001) and leukemia (Naoe et al. 2000) because NQO1 activity was not detected in the T/T genotype (Traver et al. 1997). On the other hand, our study suggests that the polymorphism (Pro187Ser) of the NQO1 gene is not associated with MAP abusers in Japan. Furthermore, it has been shown that the NQO1 gene polymorphism (Pro187Ser) is not associated with Parkinson's disease (Harada et al. 2001) and schizophrenia (Hori et al. 2003) in the Japanese samples. The lack of association of the NQO1 gene in the present study does not necessarily mean that an investigation with another sample would give the same negative result. Frequency (1.6-3.7%) of the T/T genotype in Caucasian populations (Xu et al. 2001; Sarbia et al. 2003) is lower than that (13-17.2%) of the Japanese population (Harada et al. 2001; Hori et al. 2003), suggesting the ethnic difference between Japanese and Caucasians for the NQOI gene polymorphism (Pro187Scr).

In conclusion, the present findings suggest that NQO2, but not NQO1, may contribute to the aetiology of prolonged-type MAP psychosis in the Japanese population. The sample number in this study was relatively large, but not enough. Further studies are needed to elucidate genetic vulnerability to MAP abuse, paying more attention to clinical progress and state of the disease in a larger sample of Japanese. If replication studies are confirmed, the I/D polymorphism in the promoter region of the NQO2 gene would be the known specific mechanism by which genetic variation leads to a risk for the development of MAP-induced psychosis.

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Brief report

No changes in serum epidermal growth factor levels in patients with schizophrenia

Kenji Hashimoto[a,*], Eiji Shimizu[a], Naoya Komatsu[a], Hiroyuki Watanabe[a], Naoyuki Shinoda[a], Michiko Nakazato[a], Chikara Kumakiri[a], Shin-ichi Okada[a], Nori Takei[b,c], Masaomi Iyo[a]

[a]*Department of Psychiatry, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chiba 260-8670, Japan*
[b]*Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan*
[c]*Section of General Psychiatry, Division of Psychological Medicine, Institute of Psychiatry, De Crespigny Park, London SE5 8AF, UK*

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Abstract

A recent report demonstrated that serum levels of epidermal growth factor (EGF) were significantly decreased in patients with schizophrenia, suggesting that impaired EGF signaling might be associated with the pathophysiology of schizophrenia. Our goal in the present study was to determine whether serum levels of EGF are altered in patients with schizophrenia. We found that serum levels of EGF in drug-naive ($n=15$) or medicated patients ($n=25$) with schizophrenia did not differ from those of age- and sex-matched normal controls ($n=40$). However, we found a significant correlation between serum EGF levels and BPRS scores in the combined groups of patients. Therefore, our results do not support the claim that EGF plays a role in the pathogenesis of schizophrenia, but they suggest that EGF may serve as a state marker, that is, as an index of symptom-linked deficits.

Keywords: Schizophrenia; Epidermal growth factor (EGF); Growth factor; Cytokine; Neurotrophic factor; Neurodevelopmental

1. Introduction

Schizophrenia is a devastating disorder affecting approximately 1% of the general population worldwide. The hypothesis that schizophrenia has a neurodevelopmental origin has gained much attention in the research community (Murray et al., 1992; Lewis and Lieberman, 2000). Several lines of evidence suggest that cytokines, growth factors, and neurotrophic factors may play an important role in the pathophysiology of schizophrenia (Kronfol and Remick, 2000; Buka et al., 2001; Torrey and Yolken, 2001).

* Corresponding author. Tel.: +81 43 226 2147/2149; fax: +81 43 226 2150.
E-mail address: hashimoto@faculty.chiba-u.jp (K. Hashimoto).

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In this context, of interest is a recent report showing that epidermal growth factor (EGF) protein levels are significantly decreased in the postmortem brains of patients with schizophrenia, and that serum EGF levels in both medicated and drug-free patients with the disorder are also markedly reduced as compared with normal controls (Futamura et al., 2002). In the central nervous system, EGF has been shown to serve as a neurotrophic factor to enhance cell proliferation and neuronal differentiation (Xian and Zhou, 1999), and these findings have been interpreted by the authors to be evidence that impaired EGF signaling might be associated with the pathophysiology of schizophrenia (Futamura et al., 2002). However, in the study of Futamura et al. (2002), drug-naive patients ($n=4$) and chronic patients ($n=45$; mean age: 47.0 years old) were included in the analysis of serum EGF levels. Thus, the results could be accounted for by the effect of antipsychotic medication. Furthermore, Futamura et al. (2002) did not examine the relationship between serum EGF levels and clinical variables such as the severity of symptoms in patients with schizophrenia (Futamura et al., 2002). If there was a strong relationship between serum EGF levels and the severity of patient symptoms, the pathogenetic role of EGF might be even more significant. Therefore, we measured serum EGF levels in drug-naive and medicated patients with schizophrenia and age- and sex-matched normal controls, and we also examined the possible relationship between serum EGF levels and clinical symptoms.

2. Methods

Forty patients with schizophrenia (mean: 35.5 years; 20 men and 20 women) and 40 age- and sex-matched healthy subjects (mean: 36.5 years; 20 men and 20 women) participated in this study. All of the subjects provided written informed consent for participation in the study after the procedure had been fully explained. The ethics committee of Chiba University Graduate School of Medicine approved the present study. All patients were diagnosed according to DSM-IV, and symptomatology was evaluated by a senior psychiatrist using the Brief Psychiatric Rating Scale (BPRS). Of the study patients, 15 (37.5%) were drug-naive (Table 1). Antipsychotic drugs administered to the remaining medicated patients ($n=25$) were chlorpromazine (62.5–200 mg/day; $n=3$), levomepromazine (25–100 mg/day; $n=2$), propericyazine (15 mg/day; $n=1$), fluphenazine (1.79 mg/day; $n=1$), clocapramine (150 mg/day; $n=1$), thioridazine (75 mg/day; $n=1$), haloperidol (1.5–9 mg/day; $n=5$), bromoperidol (6 mg/day, $n=1$), risperidone (3–16 mg/day; $n=16$), zotepine (25–225 mg/day; $n=3$), quetiapine (300–750 mg/day; $n=5$), or olanzapine (10–20 mg/day; $n=3$). Of the medicated patients, 12 patients were *currently* receiving multiple antipsychotic drugs for treatment. The normal controls had no history of psychiatric or neurological disorders and showed no abnormalities in routine laboratory examinations.

To minimize any time-related variations in the levels of serum EGF, serum samples were collected

Table 1
Characteristics of normal controls, drug-naive and medicated patients

	Normal controls	Drug-naive patients	Medicated patients	*P* value
Sex (M/F)	20/20	7/8	13/12	0.95[a]
Age, years (range)	36.5 ± 14.6 (20–70)	34.7 ± 16.0 (19–61)	36.0 ± 13.2 (20–65)	0.92[b]
Onset, years (range)	–	33.8 ± 15.3 (18–57)	21.2 ± 4.95 (13–35)	0.0005[c]
Illness duration, years (range)	–	1.09 ± 1.36 (0–5)	14.1 ± 9.87 (0.25–41)	<0.0001[c]
BPRS score (range)	–	26.9 ± 15.4 (6–58)	19.2 ± 12.4 (2–56)	0.09[c]
Chlorpromazine equivalents (mg)	–	–	727 ± 412 (113–1900)	
EGF (pg/ml)	411 ± 217	331 ± 226	481 ± 241	0.13[b]

Age, onset, illness duration, and BPRS score were shown in the mean ± SD (range).
The value of EGF was shown in the mean ± SD.
[a] The comparison among three groups was performed by chi-square test.
[b] The comparison among three groups was performed by ANOVA.
[c] The comparison between two groups was performed by *t*-test.

from the patients and normal controls between 11:00 and 12:00 h and stored at −80 °C until assay. Serum EGF levels were determined with a commercial quantitative sandwich enzyme immunoassay technique (Quantikine® EGF immunoassay kit, R&D Systems, Inc., Minneapolis, MN, USA). All analyses and calibrations were carried out in duplicate, and each plate included recombinant human EGF. The intra-assay coefficient of variation (CV) was 2%, and the inter-assay CV was 4%.

The chi-square test was used for categorical variables, and the Student *t*-test and analysis of variance (ANOVA) were employed for continuous variables since the data were normally distributed. The relationship between two variables was examined using Pearson's correlation coefficients. Significance for the results was set at $P<0.05$.

3. Results

Age- and sex-matching was found to be successful, as there was no marked or significant difference among the three test groups (normal controls, drug-naive patients, and medicated patients) (Table 1). One-way ANOVA showed no overall significant ($F=2.10$, $df=2, 77$, $P=0.13$) difference in the serum EGF levels among the three groups [normal controls ($n=40$): 411 ± 217 pg/ml; drug-naive patients ($n=15$): 331 ± 226 pg/ml; medicated patients ($n=25$): 481 ± 241 pg/ml]. There was no correlation between the serum EGF levels and duration of illness in the drug-naive patients or medicated patients.

We then examined the correlation between serum EGF levels and BPRS score in the drug-naive and medicated patients with schizophrenia and found a significant positive correlation in the combined groups of patients ($r=0.434$, $P=0.005$). These positive correlations were present for both the drug-naive ($r=0.595$, $P=0.018$) and medicated patients ($r=0.549$, $P=0.004$). The significant correlation in the medicated patients could be accounted for by the antipsychotic medication. Patients with a more severe form may receive a higher dose of medication. In fact, although there was no overall group difference, serum EGF levels were slightly higher in the medicated patients than in the normal controls and drug-naive patients. Even when the antipsychotics taken (chlorpromazine equivalents) were adjusted for by using partial correlation coefficients, the relationship between the BPRS scores and serum EGF levels in the medicated patients remained significant (partial correlation coefficient=0.497, $P=0.013$). In addition, we found a significant ($r=0.487$, $P=0.013$) correlation between total BPRS scores and antipsychotic dose (chlorpromazine equivalents) in the medicated patients. In contrast, there was no significant correlation ($r=0.269$, $P=0.196$) between the antipsychotic dose (chlorpromazine equivalents) and serum EGF levels in the medicated patients.

4. Discussion

In the present study, we found that serum EGF levels in drug-naive and medicated patients with schizophrenia were not different from those of normal controls. These findings contrast with those of a report (Futamura et al., 2002) showing that serum EGF levels in both drug-naive ($n=4$) and medicated chronic patients ($n=45$) were lower than those in normal controls. Our sample size of drug-naive patients ($n=15$) is much larger than that ($n=4$) in the study by Futamura et al. (2002). Consequently, our results do not support the claim (Futamura et al., 2002) that EGF plays an important role in the pathogenesis of schizophrenia. Further detailed studies are needed to determine whether serum EGF serves as a marker for susceptibility to schizophrenia.

In contrast, we found that serum EGF levels correlated significantly with the severity of symptoms as assessed using the BPRS in both drug-naive and medicated patients with schizophrenia. Furthermore, we found a significant correlation between BPRS scores and antipsychotic dose (chlorpromazine equivalents) in the medicated patients. Thus, it seems that EGF may serve as a state marker, that is, as an index of symptom-linked deficits, although further detailed studies are needed.

In conclusion, our results do not support the claim that EGF plays an important role in the pathogenesis of schizophrenia, but they suggest that EGF may serve as a state marker, that is, as an index of symptom-linked deficits.

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Functional Polymorphism of the Glutathione Peroxidase 1 Gene Is Associated with Personality Traits in Healthy Subjects

Daisuke Matsuzawa Kenji Hashimoto Eiji Shimizu Mihisa Fujisaki
Masaomi Iyo

Department of Psychiatry, Chiba University Graduate School of Medicine, Chiba, Japan

Key Words
Glutathione peroxidase 1 · Polymorphism · Personality · Temperament · Antioxidant

Abstract
Background: Several lines of evidence suggest that a certain type of personality or temperament as well as oxidative stress may be implicated in the pathophysiology of neuropsychiatric diseases. Glutathione peroxidase 1 (GPX1) plays a role in the antioxidant defense system. ***Objectives:*** The authors studied the association between the GPX1 gene polymorphism and personality traits in healthy subjects. ***Methods:*** One hundred forty-nine healthy subjects were enrolled. Analysis of the functional polymorphism (Pro198Leu) in the human GPX1 gene was performed. ***Results:*** Subjects with Pro198Pro have significantly higher scores in openness to experience on the Revised NEO Personality Inventory (NEO-PI-R) as compared with subjects with other genotypes (Pro198Leu or Leu198Leu). In contrast, we detected no association between other personality dimensions on the NEO-PI-R and scores on the Temperament and Character Inventory. ***Conclusion:*** This study reports that the functional polymorphism (Pro198Leu) in the GPX1 gene might be associated with openness to experience among the personality traits.

Introduction

Several lines of evidence suggest that a certain type of personality and temperament may increase a risk for neuropsychiatric diseases such as mood disorders [1, 2], and other reports have implicated oxidative stress in the pathophysiology of neuropsychiatric diseases including mood disorders [3]. Glutathione peroxidases protect cells against oxidative damage by reducing hydrogen peroxide and a wide range of organic peroxides with reduced glutathione. Glutathione peroxidase 1 (GPX1), which is widely distributed in human cells and belongs to a family of selenium-dependent peroxidases, plays a major role in the antioxidative defense process. GPX1 eliminates peroxides by reducing them to H_2O or alcohols, with glutathione as reducing substrates [4]. The human GPX-1 gene is located on the chromosome 3p21 locus and contains two exons. A single nucleotide polymorphism (SNP) in the human GPX1 gene at nucleotide 594, a C→T substitution (db SNP ID: rs1050450), causes a proline (Pro) to leucine (Leu) substitution at codon 198 (Pro198Leu) [5]. The GPX1 activity associated with the protein by the Leu-containing allele is less responsive to added selenium than it is with the same protein containing a Pro at that position [6], suggesting a functional consequence to this allelic variation.

Considering the role of oxidative stress in neuropsychiatric diseases, it is of great interest to examine the as-

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Dr. Kenji Hashimoto
Department of Psychiatry, Chiba University Graduate School of Medicine
1-8-1 Inohana
Chiba 260-8670 (Japan)
Tel. +81 43 226 2147, Fax +81 43 226 2150, E-Mail hashimoto@faculty.chiba-u.jp

Table 1. Association between the GPX1 gene polymorphism and NEO-PI-R scores and TCI scores in healthy subjects

Genotype	NEO-PI-R scores				
	neuroticism	extraversion	openness to experience	agreeableness	consciousness
C/C (n = 124)	103.9 (22.4)	104.2 (21.1)	119.6 (15.0)*	110.3 (15.8)	100.9 (19.0)
C/T (n = 23)	100.3 (17.8)	99.5 (15.1)	109.3 (15.2)*, **	116.7 (11.9)	104.4 (13.2)
T/T (n = 2)	100.5 (9.19)	92.0 (12.7)	97.0 (5.66)*, ***	120.0 (5.66)	91.5 (12.0)

Genotype	TCI scores						
	NS	HA	RD	P	SD	C	ST
C/C (n = 124)	21.6 (5.47)	18.5 (6.49)	16.2 (6.49)	4.44 (2.08)	28.7 (6.81)	28.9 (6.17)	10.9 (5.62)
C/T (n = 23)	19.4 (4.91)	20.2 (5.01)	16.4 (3.95)	4.43 (1.64)	26.8 (6.73)	29.4 (3.71)	10.9 (5.28)
T/T (n = 2)	20.5 (0.71)	21.5 (2.12)	15.5 (2.12)	5.00 (1.41)	19.5 (2.12)	24.0 (4.24)	10.0 (4.24)

NS = Novelty seeking; HA = harm avoidance; RD = reward dependence; P = persistence; SD = self-directedness; C = cooperativeness; ST = self-transcendence. Data are shown as means, with SD in parentheses. * $p = 0.002$ (ANOVA); ** $p = 0.008$ (vs. C/C genotype); *** $p = 0.03$ (vs. C/C genotype).

sociation between the GPX1 gene polymorphism and personality traits. In the present study, we examined the association between the functional (Pro198Leu) polymorphism of the GPX1 gene and personality trait scores assessed on the Temperament and Character Inventory (TCI) and the Revised NEO Personality Inventory (NEO-PI-R) in healthy Japanese subjects.

Materials and Methods

We recruited 149 Japanese healthy subjects. Subjects consisted of 65 males (mean ± SD 26.6 ± 5.0 years, range 22–43 years) and 84 females (mean ± SD 28.2 ± 11.3 years, range 20–58 years). All subjects were instructed to complete self-report inventories using the Japanese version of the NEO-PI-R and the TCI [7, 8]. This study was approved by the ethics committee of Chiba University Graduate School of Medicine. Genomic DNA was extracted from blood samples obtained after completing written informed consent.

The Pro198Leu polymorphism on the GPX1 gene was assayed using the PCR restriction fragment length polymorphism technique, as reported by Hu and Diamond [6]. Extracted DNA samples were amplified by PCR with the following primers: forward: 5′-TGTGCCCCTACGGTA-3′, and reverse: 5′-CCAAATGACAATGACACAGG-3′. Digestion was performed with restriction enzyme *Apa*I (New England Biolabs, Beverly, Mass., USA), and samples were electrophoresed on 2% agarose gel.

A χ^2 test was used for categorical comparisons, and Student's t test was employed for age difference. Differences among the three groups were examined by means of one-way analysis of variance (ANOVA), followed by the Bonferroni post hoc test. Significance for the results was set at $p < 0.05$.

Results

The frequencies of C and T alleles of the GPX1 gene were 90.9 and 9.1%, respectively. The frequencies of C/C, C/T, and T/T were 83.2, 15.4, and 1.3%, respectively. The genotype distribution was within the Hardy-Weinberg equilibrium. No gender differences in the distribution of genotypes ($\chi^2 = 0$ 3.58, $p = 0.177$) or alleles ($\chi^2 = 2.95$, $p = 0.104$) were detected. ANOVA revealed significant differences among the three groups ($F = 6.455$, d.f. = 148, $p = 0.002$) in the scores of openness to experience on the NEO-PI-R; the scores of openness to experience on the NEO-PI-R in the C/T genotype ($p = 0.003$) and T/T genotype ($p = 0.03$) were significantly lower than those in the C/C genotype (table 1). In contrast, ANOVA revealed no difference among the three groups in the scores of neuroticism, extraversion, agreeableness, or conscientiousness (table 1). Furthermore, no significant difference among the three groups was detected in four dimensions of temperament (novelty seeking, harm avoidance, reward dependence, and persistence) or three dimensions of character (self-directedness, cooperativeness, and self-transcendence) on the TCI (table 1).

Discussion

This study shows that groups with a GPX1 heterozygous genotype (Pro198Leu) or homozygous genotype (Leu198Leu) have significantly lower scores in openness to experience on the NEO-PI-R compared with the homozygous (Pro198Pro) group, suggesting that this functional polymorphism of the GPX1 gene is associated with specific personality traits in healthy subjects. As to neuropsychiatric disorders, an association study between GPX1 gene Pro198Leu polymorphism and schizophrenia was reported although no positive association was shown [9]. However, several reports demonstrated that lower antioxidant defense against lipid peroxidation exists in patients with depression, and there is a therapeutic benefit from antioxidant supplementation in unstable manic-depressive patients. GPX1 activity during the pretreatment period in the patients with mood disorders was significantly lower than that of both posttreatment patients and control groups [10], suggesting that the antioxidant system might be impaired during a mood episode in patients with mood disorders, normalizing at the end of the episode. Openness to experience is seen as aesthetic sensitivity, intellectual curiosity, need for variety, and a nondogmatic attitude. Scores in openness to experience on the NEO-PI-R in patients with unipolar depression are shown to be lower than those in patients with bipolar disorder [11]. Furthermore, it has been reported that two facets [aesthetics (O2) and feelings (O3)] on openness to experience are associated with depression [11, 12] and that the feelings (O3) facet is significantly associated with the brain-derived neurotrophic factor (BDNF) gene Val66Met polymorphism, while the aesthetics (O2) facet shows a trend towards an association with the BDNF gene polymorphism [13]. Given the role of BDNF in the pathophysiology of mood disorders [2], it should be noted that the BDNF gene polymorphism is associated with personality traits such as openness to experience. Taken together, based on the role of GPX1 in the antioxidant defense system, it seems that subjects with the T allele have lower GPX1 activity, with the result that those subjects have a weaker antioxidant defense system than do subjects with the C allele. It is, therefore, likely that the functional polymorphism of the GPX1 gene may play a role in the pathogenesis of mood disorders. Because of the relatively small sample size, our findings in this study should be limited. Further study using a large sample would be necessary.

In conclusion, we found an association between the Pro197Leu polymorphism in the GPX1 gene and the personality trait of openness to experience on the NEO-PI-R in healthy Japanese subjects. To our knowledge, this is the first study demonstrating that the functional polymorphism of the antioxidant defense enzyme GPX1 gene is associated with personality traits in healthy subjects, suggesting that this polymorphism may be implicated in the pathogenesis of mood disorders.

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BRIEF REPORT

Positive association of AKT1 haplotype to Japanese methamphetamine use disorder

Masashi Ikeda[1,2], Nakao Iwata[1,3], Tatsuyo Suzuki[1], Tsuyoshi Kitajima[1], Yoshio Yamanouchi[1], Yoko Kinoshiya[1], Yoshimoto Sekine[3,4], Masaomi Iyo[3,5], Mutsuo Harano[3,6], Tokutaro Komiyama[3,7], Mitsuhiko Yamada[3,8], Ichiro Sora[3,9], Hiroshi Ujike[3,10], Toshiya Inada[2,3] and Norio Ozaki[2,3]

[1] *Department of Psychiatry, Fujita Health University School of Medicine, Aichi, Japan*
[2] *Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Japan*
[3] *Japanese Genetics Initiative for Drug Abuse (JGIDA), Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*
[4] *Department of Psychiatry and Neurology, Hamamatsu University school of Medicine, Hamamatsu, Japan*
[5] *Department of Psychiatry, Graduate School of Medicine, Chiba University, Chiba, Japan*
[6] *Department of Neuropsychiatry, Kurume University School of Medicine, Kurume, Japan*
[7] *Division of Psychiatry, National Center Hospital for Mental, Nervous and Muscular Disorders, National Center of Neurology and Psychiatry, Tokyo, Japan*
[8] *Division of Psychogeriatrics, National Institute of Mental Health, National Center of Neurology and Psychiatry, Chiba, Japan*
[9] *Department of Neuroscience, Division of Psychobiology, Tohoku University Graduate School of Medicine, Sendai, Japan*
[10] *Department of Neuropsychiatry, Okayama University Graduate School of Medicine and Dentistry, Okayama, Japan*

Abstract

Recent evidence suggests that the AKT1-GSK3β signalling cascade partially mediates dopamine-dependent behaviours. In relation to the pathophysiology of schizophrenia or methamphetamine (Meth) use disorder, AKT1 is a good candidate gene for such conditions. For schizophrenia, positive associations of SNPs and AKT1 haplotypes were reported in US and Japanese samples. To evaluate the association between AKT1 and Meth-use disorder, we conducted a case-control study of Japanese samples (182 patients and 437 controls). A positive association between a SNP and haplotypes was found, and the 'signal' SNP was the same SNP found to be associated with US schizophrenia, but not with Japanese schizophrenia. Our results indicate that AKT1 may play a possible role in the development of Meth-use disorder. Further investigation of these associations, together with evidence from previous animal studies, may open the way to elucidation of the pathophysiology of this condition.

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Key words: Dopamine-dependent behaviours, linkage disequilibrium, substance-related disorders.

Introduction

The pathophysiology of methamphetamine (Meth) use disorder has not been well established, however, one of the most likely mechanisms is abnormality of the dopamine (DA) neurotransmission system. The pharmacological profile of Meth shows that the target site of Meth is the DA transporter (DAT). Also the mesolimbic DA system has an important function in reinforcement and reward mechanisms (Spanagel and Weiss, 1999).

Address for correspondence: N. Iwata, M.D., Ph.D., Department of Psychiatry, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan.
Tel.: +81-562-93-9250 *Fax*: +81-562-93-1831
E-mail: nakao@fujita-hu.ac.jp

Family and twin studies suggested that the genetic contribution is important in that it may predispose certain people to this disorder (Tsuang et al., 1996, 1998). Recent studies have suggested that V-akt murine thymoma viral oncogene homologue 1 (AKT1) is a good candidate for a Meth-use disorder susceptibility gene, for the following reasons. (1) An animal study of DAT knock-out (KO) mice and wild-type mice, treated with lithium salts and amphetamine, showed that the AKT1–glycogen synthase kinase 3β (GSK3β) signalling cascade partially mediated DA-dependent behaviours (Beaulieu et al., 2004). (2) AKT1 KO mice treated with amphetamine showed a reduction in prepulse inhibition (PPI) (Emamian et al., 2004). PPI disturbances are known to be present in schizophrenia, which might also be related to

abnormalities in the DA system. AKT1 haplotypes were shown to have a significant association with schizophrenia in a transmission disequilibrium test (TDT) study (Emamian et al., 2004) and in a previous Japanese case-control replication study by the authors (Ikeda et al., 2004), although no association was found in another Japanese replication study (Ohtsuki et al., 2004).

Here we conducted a case-control study of Japanese Meth-use disorder samples using the single nucleotide polymorphisms (SNPs) of our previous study to evaluate the association of AKT1 with Meth-use disorder.

Methods

A total of 182 patients with Japanese Meth-use disorder [146 male, 36 female; mean age ± standard deviation (S.D.), 36.7 ± 12.0 yr] and 437 controls (209 male, 228 female; 34.3 ± 13.6 yr) were analysed. The number of patients with Meth-use disorder comprised of 168 Meth-dependent subjects, and 14 Meth-abuse subjects. Among the subjects with Meth-use disorder, 153 subjects (127 males, 26 females) have a comorbid diagnosis of Meth-induced psychosis, three of anorexia nervosa, one of obsessive–compulsive disorder, and one of major depressive disorder. And 120 subjects with Meth-use disorder have abuse or dependence on drugs other than Meth. Subjects with Meth-use disorder were excluded if they had a comorbid diagnosis of any psychotic disorder other than Meth-induced psychosis. They were diagnosed according to DSM-IV criteria by the consensus of at least two experienced psychiatrists on the basis of unstructured interviews and review of the medical records. All healthy controls were also psychiatrically screened based on unstructured interviews. More details about the characterization of these subjects have been published elsewhere (Suzuki et al., 2003; Ujike et al., 2003). After description of the study, written informed consent was obtained from each subject. This study was approved by the Ethics Committee at each participating institute of the Japanese Genetics Initiative for Drug Abuse (JGIDA).

For SNP genotyping, polymerase chain reaction (PCR) amplification, restriction fragment length polymorphism (RFLP) assays were developed; *Bsa*I for SNP1 (rs3803300), *Xcm*I for SNP2 (rs1130214), *Hae*III for SNP3 (rs3730358), *Hpy*CH4IV for SNP4 (rs2498799), *Pst*I for SNP5 (rs2494732), and *Bsi*HKAI for SNPA (rs2498804). A detailed description may be found in a previous report (Ikeda et al., 2004) and information about primer sequences and PCR–RFLP conditions are available on request.

Hardy–Weinberg equilibrium (HWE) was evaluated by conventional χ^2 test (SPSS 10.0J, SPSS Japan Inc., Tokyo, Japan). For marker-trait association analyses, we constructed multi-SNP haplotype systems (Emamian et al., 2004) to evaluate the association through permutation p values in sliding window fashion and global p values respectively. In total sample association analysis (not in explorative association analysis), we emphasize the permutation p values over the respective global p values because the permutation procedure gives a significance corrected for the multiple haplotypes and markers tested. Furthermore, we corrected these permutation p values by Bonferroni correction to obtain more robust results. A more detailed description is given in our previous paper (Ikeda et al., 2004).

We also include an explorative analysis for gender effects, because of the following reasons. (1) Aetiological study suggests that the genetic contribution of substance-related disorder is differentially heritable by gender (Jang et al., 1997). (2) Our samples were unmatched gender ratios of Meth-use disorder (36 female, 146 male).

Results

Genotype frequencies of all SNPs were in HWE. Positive permutation p values of 4- and 5-marker sliding window fashion ($p = 0.0083$ and 0.023 respectively) and global p value of 6-marker combinations ($p = 0.017$) were obtained. One of the 4-marker sliding window fashion p values remained significant ($p = 0.0498$) even after Bonferroni correction was performed six times (once for single marker permutation and five times for haplotype combinations). In the single marker association analysis (i.e. a conventional allele-wise association analysis), only SNP3 was associated with Meth-use disorder ($p = 0.019$) (Table 1).

Individual haplotypic analyses from the positive global 4-marker p values are shown in Table 2. The haplotype with the most significant association was more frequent in controls than in cases (SNP1-2-3-4, G-G-C-G, $p = 0.0032$).

Explorative analysis of gender effects is shown in Table 3. In female samples, eight of 21 global p values showed significance. In these significant p values, SNP3, which was associated with total Meth-use disorder, showed strong association ($p = 0.0011$). On the other hand, the positive global p values in male samples tended to be similar to those in total samples (positive global p values: SNP1-2-3-4 = 0.036, SNP1-2-3-4-5-A = 0.042), however, SNP3 was not associated with male Meth-use disorder ($p = 0.11$).

Table 1. Association analyses of the AKT1 gene

							Genotypic distribution					
	Multi-SNP haplotype systems						M/M		M/m		m/m	
SNP ID	1 SNP	2 SNP	3 SNP	4 SNP	5 SNP	6 SNP	Meth	Control	Meth	Control	Meth	Control
SNP1 (rs3803300G>A)	0.15						63	124	91	234	28	79
		0.22										
SNP2 (rs1130214G>T)	0.97		0.096				128	315	51	108	3	14
		0.27		**0.0023**								
SNP3 (rs3730358C>T)	**0.019**		0.43		**0.0082**		136	364	43	68	3	5
		0.12		0.23		**0.017** (0.10)						
SNP4 (rs2498799G>A)	0.81		0.11		0.16		40	121	98	211	44	105
		0.53		0.063								
SNP5 (rs2494732A>G)	0.59		0.19				86	212	79	192	17	33
		0.16										
SNPA (rs2498804T>G)	0.20						63	142	92	206	27	89
Permutation *p* value	*0.097*	*0.40*	*0.28*	***0.0083*** (***0.0498***)	***0.023*** (*0.14*)							

p values were calculated by log likelihood ratio test (SNP1, allele-wise association; SNP2-6, global haplotypic association).
M, major allele; m, minor allele; Meth, methamphetamine-use disorder.
Bold values represent significant *p* values.
Values within parentheses represent *p* values after Bonferroni correction.

Table 2. Haplotype frequencies from positive permutation analysis

Combination of SNPs	Marker haplotype	Frequency		*p* values
		Meth	Control	
SNP1-2-3-4	A-G-C-G	0.28	0.20	0.023
	A-G-T-A	0.074	0.048	0.049
	G-G-C-G	0.12	0.21	0.0032

Meth, Methamphetamine-use disorder.

Discussion

A positive association between a SNP and AKT1 haplotypes was found in our Japanese Meth-use disorder samples. In assessing the components of these associations, we considered SNP3 to be a main component associated with Meth-use disorder, because the single marker association of SNP3 was significant in total samples ($p=0.019$). Interestingly, this SNP was associated with US schizophrenia in an original TDT analysis (Emamian et al., 2004). On the contrary, we found no association SNP3 to Japanese schizophrenia in a previous study (SNP5 was associated with Japanese schizophrenia) (Ikeda et al., 2004). This difference in predisposing SNPs between Japanese Meth-use disorder and Japanese schizophrenia might be explained by their respective linkage disequilibrium (LD) patterns. We have shown that the LD pattern in schizophrenia was slightly different from that in controls, while the pattern in Meth-use disorder tended to be similar to that in control samples (data not shown). These findings indicate that different predisposing polymorphisms may exist independently in schizophrenia and Meth-use disorder, and may be located in LD with SNP5 or SNP3 respectively.

The result of explorative analysis might support the 'gender effects' of Meth-use disorder, reported in a previous genetic association study of Meth-use disorder (Lin et al., 2003). Especially, female samples of

Table 3. Explorative analysis of gender effects

		Multi-SNP haplotype systems					
Gender	SNP ID	1 SNP	2 SNP	3 SNP	4 SNP	5 SNP	6 SNP
Female ($n=36$)	SNP1	0.55					
			0.41				
	SNP2	0.084		**0.017**			
			0.012		**0.012**		
	SNP3	**0.0011**		**0.049**		**0.028**	
			0.028		0.084		**0.023**
	SNP4	0.50		0.076		0.057	
			0.67		0.16		
	SNP5	0.34		0.79			
			0.57				
	SNPA	0.73					
Male ($n=146$)	SNP1	0.23					
			0.13				
	SNP2	0.28		0.18			
			0.51		**0.036**		
	SNP3	0.11		0.47		0.053	
			0.26		0.41		**0.042**
	SNP4	0.58		0.19		0.22	
			0.55		0.078		
	SNP5	0.40		0.12			
			0.30				
	SNPA	0.50					

Bold values represent significant p values.

Meth-use disorder were strongly associated with a SNP and haplotypes of AKT1, while male samples were weakly associated. However, because the sample size of female subjects was small ($n=36$), a type I error might occur in this explorative analysis. Even assuming that there are no 'gender effects' of AKT1, the fact remains that AKT1 is associated with Meth-use disorder. In this case, these association analyses of total and divided samples indicate the following interpretations. (1) SNP3 might not be an 'actual' predisposing SNP by itself, nor be in perfect LD with 'actual' predisposing polymorphisms, because male samples with Meth-use disorder were not associated with SNP3 (only total or female samples were associated with it). (2) At least some haplotypes of AKT1 may play a possible role in the development of Meth-use disorder, because two haplotypes of AKT1, including the combination of SNP1-2-3-4 and SNP 1-2-3-4-5-A, are associated with Meth-use disorder both in divided samples and total samples.

Our results had several limitations in terms of interpreting positive associations. (1) The positive associations we detected might be due to type I error, possibly because of population stratification, an unmatched-gender sample and small sample size, described above. Larger sample size and genomic control would be required. (2) Type I error might also have occurred because of multiple testing. We corrected p values by applying a permutation procedure and Bonferroni correction in total samples. However, in individual haplotypic analysis or explorative analysis of gender effects, we did not apply each correction. At this time, an optimal method for resolving this problem (correction among global and individual haplotypic analysis, or explorative-subgroup analysis) has not yet been established. Greater knowledge of genetic models and more useful methods would be required to re-analyse these results. (3) The other confounding factors such as ascertainment bias can account for the apparent association in this study. For example, because subjects with Meth-induced psychosis consisted of the majority of our samples, this condition would be over-represented in our samples of Meth-use disorder. Further explanation is given in Nishiyama et al. (2005).

Our results indicate that AKT1 may play a role in the development of Meth-use disorder. Our findings also support the hypothesis that abnormalities in AKT1 might contribute to the pathophysiology of DA-dependent behaviour such as Meth-use disorder and schizophrenia. Further studies including mutation search to detect 'actual' predisposing polymorphisms and functional analysis of AKT1 (or its cascade) may open the way to elucidation of the pathophysiology of this condition.

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Statement of Interest

None.

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Identification of Multiple Serine Racemase (*SRR*) mRNA Isoforms and Genetic Analyses of *SRR* and *DAO* in Schizophrenia and D-Serine Levels

Kazuo Yamada, Tetsuo Ohnishi, Kenji Hashimoto, Hisako Ohba, Yoshimi Iwayama-Shigeno, Manabu Toyoshima, Akira Okuno, Hitomi Takao, Tomoko Toyota, Yoshio Minabe, Kazuhiko Nakamura, Eiji Shimizu, Masanari Itokawa, Norio Mori, Masaomi Iyo, and Takeo Yoshikawa

Background: *We previously reported a reduction in serum levels of D-serine, an endogenous co-agonist of the N-methyl-D-aspartate (NMDA) receptor, in schizophrenia, supporting the hypofunction hypothesis of NMDA neurotransmission in schizophrenia. In this study, we examined the genetic roles of serine racemase (SRR), an enzyme catalyzing the formation of D-serine from L-serine, and D-amino-acid oxidase (DAO) in the susceptibility to schizophrenia and the regulation of serum D-serine levels.*

Methods: *We determined the complete cDNA and genomic structures of SRR and performed mutation screening. Single nucleotide polymorphisms (SNPs) in SRR and DAO were tested for their association with schizophrenia in both case–control and family-based designs and for correlation with serum levels of D-serine.*

Results: *Genomic analyses revealed that human brain SRR transcripts consist of four isoforms with one major species, which were derived from alternative use of various 5' end exons. Genetic association analyses showed no significant association between SRR/DAO and schizophrenia. We replicated the decreased serum D-serine levels in schizophrenia in the sample set, but D-serine levels did not correlate with SRR/DAO genotypes.*

Conclusions: *The SRR/DAO are not likely to be major genetic determinants in the development of schizophrenia or control of serum D-serine levels.*

Key Words: Alternative splicing, genotype–phenotype correlation, glycine site, mutation screening, N-methyl-D-aspartate receptor, polymorphism.

The precise etiology of schizophrenia remains largely unknown, and the genetic determinants of the disease are complex, making identification of definitive susceptibility genes a formidable task (Gottesman 1991; Kendler 2005). Converging evidence, including genetic studies, has, however, supported the hypofunction hypothesis of glutamatergic neurotransmission via the N-methyl-D-aspartate (NMDA)-type glutamate receptor in schizophrenic brains. This hypothesis originally stemmed from clinical observations that phencyclidine and its congener anesthetic ketamine, both acting as noncompetitive antagonists of the receptor, evoke a schizophrenia-like psychosis including positive and negative symptoms in healthy control subjects and that phencyclidine exacerbates schizophrenic symptoms in patients (Javitt and Zukin 1991; Krystal et al 1999). The cognate NMDA receptor is heteromeric, consisting of the indispensable NR1 subunit and one of six possible subunits, NR2A, NR2B, NR2C, NR2D, NR3A, or NR3B (Andersson et al 2001; Dingledine et al 1999). Genetic variations in the receptor molecules themselves (Itokawa et al 2003; Miyatake et al 2003), and other molecules that may indirectly influence NMDA receptor function (for review, see Harrison and Owen 2003) have been reported to confer a risk for schizophrenia.

From the Laboratory for Molecular Psychiatry (KY, TO, HO, YI-S, MT, AO, HT, TT, TY), RIKEN Brain Science Institute, Wako, Saitama; Department of Psychiatry (KH, ES, MIy), Chiba University Graduate School of Medicine, Chiba; Department of Psychiatry and Neurology (YM, KN, NM), Hamamatsu University School of Medicine, Hamamatsu; Department of Schizophrenia Research (MIt), Tokyo Institute of Psychiatry, Tokyo, Japan.

Address reprint request to Takeo Yoshikawa, M.D., Ph.D., Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, 2-1 Hirosawa, Wako-city, Saitama 351-0198, Japan; E-mail: takeo@brain.riken.go.jp.

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The receptor forms a cation channel, the opening of which is modulated by an arosteric glycine-binding site (Danysz and Parsons 1998; Leeson and Iversen 1994). The endogenous ligands for this strychnine-insensitive glycine site are deemed to include D-serine (Snyder and Ferris 2000). Importantly, therapeutic trials with D-serine have been shown to improve the positive and negative symptoms, and cognitive deficit, of patients with schizophrenia (Tsai et al 1998). These results suggest that endogenous D-serine could play an imperative role in the pathophysiology of schizophrenia (Goff and Coyle 2001). D-serine is synthesized by a glial serine racemase (SRR), a novel pyridoxal-5'-phosphate (vitamin B6)-dependent enzyme converting L-serine to D-serine in the mammalian brain (Schell 2004; Snyder and Ferris 2000; Wolosker et al 1999a, 1999b). Degradation of D-serine is mediated by D-amino acid oxidase (DAO), but this enzyme is not present in forebrain areas that are highly enriched for D-serine (Hashimoto et al, in press; Nagata 1992; Schell 2004). It is also known that glycine is converted to L-serine by the pyridoxal-5'-phosphate-dependent enzyme, serine hydroxymethyltransferase (Bauwe and Kolukisaoglu 2003). We recently reported that serum levels of D-serine and the ratio of D-serine to total serine were indeed significantly decreased in schizophrenia patients, suggesting that the activity of SRR may be reduced in schizophrenia (Hashimoto et al 2003). In this study, we first clarified the genomic architecture of the human *SRR* gene and then examined the genetic role of this gene in the susceptibility to schizophrenia and regulation of serum D-serine levels. We also studied the genetic contribution of the DAO, which catalyzes the oxidative deamination of D-amino acids, with the exception of D-aspartate and D-glutamate (which are oxidized by D-aspartate oxidase; Sacchi et al 2002), to the previously mentioned phenotypes.

Methods and Materials

Subjects

Fifty patients with schizophrenia (mean age 36.9 ± 14.2 years) and 52 healthy control subjects (mean age 30.3 ± 8.0

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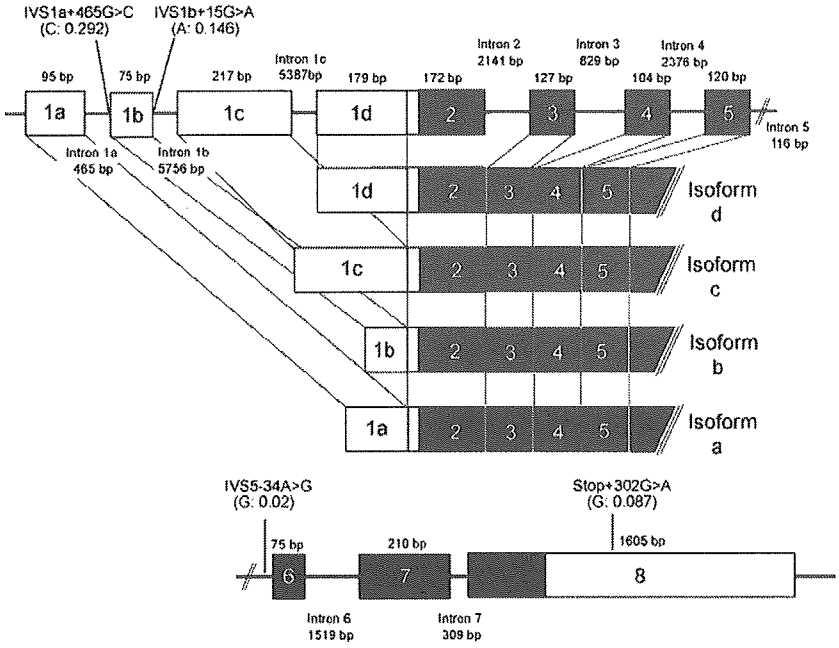

Figure 1. Genomic structure, transcript isoforms, and location of polymorphic sites for human *SRR*. Exons are denoted by boxes, with untranslated regions in white and translated regions in black. Note that the alternative use of 5′ end untranslated exons of 1a, 1b, 1c, and 1d generates four mRNA isoforms, of which isoform b is the major transcript in the brain.

years; hereafter referred to as sample set A) were used for evaluation of both serum levels of D and L serines and genotypes. Among these subjects, 31 schizophrenia patients (62%) and 44 normal control subjects (85%) were newly recruited in this study; the remainder were the same as those analyzed previously (Hashimoto et al 2003).

For a large-scale case–control genetic association study, an independent sample panel (referred to as sample set B) was used, which comprised 570 unrelated schizophrenia patients (285 men, 285 women; mean age 47.0 ± 11.4 years) and 570 age- and gender-matched control subjects who showed no history of mental illness in a brief psychiatric interview (285 men, 285 women; mean age 46.7 ± 11.1 years).

The third independent sample panel (referred to as sample set C), which was used for a family-based association test, consisted of 124 families with 376 members, of whom 163 were affected. This included 80 independent and complete trios (schizophrenic offspring and their parents), 15 probands with one parent, 13 probands with affected siblings, and 30 probands with discordant siblings.

All subjects resided in central Japan. A consensual diagnosis was made according to DSM-IV by at least two experienced psychiatrists on the basis of direct interviews, available medical records, and information from hospital staff and relatives. None of the patients had additional Axis I disorders as defined by DSM-IV.

The study was approved by the ethics committees of RIKEN and Chiba University Graduate School of Medicine. All control subjects and patients and family members gave informed written consent to participate in the study after provision and explanation of study protocols and purposes.

5′-RACE (Rapid Amplification of cDNA Ends) and Determination of the Genomic Structure for SRR

A partial cDNA sequence for *SRR* was obtained from GenBank (accession No. NM_021947) and the full length transcript was isolated by 5′-RACE using a brain-derived Marathon cDNA kit (BD Biosciences Clontech, Palo Alto, California), according to

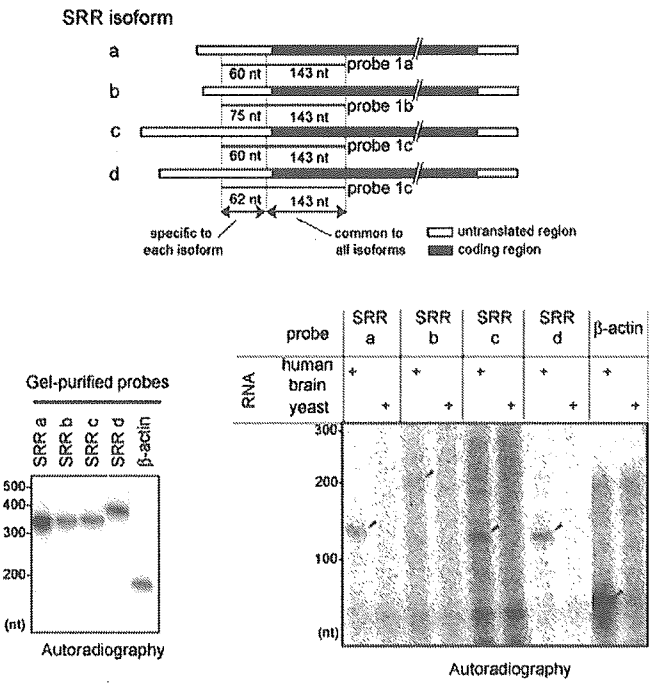

Figure 2. RNase protection assay of *SRR* transcripts. Upper panel shows *SRR* mRNA isoforms and positions of specific probes (probes 1a, 1b, 1c, and 1d). Filled boxes show the coding region of mRNA and open boxes indicate 5′- or 3′-untranslated regions. Note that all four probes consist of an isoform-specific region (60–75 nt) and common region (143 nt). Each probe is flanked by 71 nt at the 5′ end and 41 nt at 3′ end with vector derived sequences. The lower left panel is an autoradiogram, showing the integrity of radio-labeled riboprobes. Probes for *SRR* isoforms a–d and β-actin were gel-purified after synthesis and re-electrophoresed in a denaturing gel to evaluate the integrity of the probes. The lower right panel shows the results of the RNase protection assay. Each probe was hybridized to human total RNA or yeast total RNA (negative control), and then an RNase protection assay was performed. Arrowheads in the autoradiogram show the positions of specific signals.

Table 1. PCR Primers Used to Search for Nucleotide Variants in the SRR Gene

Region	Primers	Product Size (bp)	5′ End of Primer	Polymerase and Buffer
5′ Upstream of Exon 1a	(F) 5′-CTATTGCCTAACGCAGGAGAGGT-3′ (R) 5′-TCCTGCCTCCACCCTCTTTC-3′	440	−12593 (415 bp Upstream of Exon 1a) −12154 (Exon 1a)	Taq and Master Amp K
Exon 1a	(F) 5′-GGCGGCCTGGAGAGCGATA-3′ (R) 5′-TTCCCCCAGCAGTAGGCG-3′	267	−12301 (Upstream of Exon 1a) −12035 (Intron 1a)	Taq and Master Amp K
5′ Upstream of Exon 1b	(F) 5′-AGAAAGAGGGTGGAGGCAGG-3′ (R) 5′-CTGTCAACCCGAGTCCCAGA-3′	741	−12174 (Exon 1a, 556 bp Upstream of Exon 1b) −11434 (Intron 1b)	Taq and Master Amp K
Exon 1b	(F) 5′-TGGCCGCGCTGGGAGGAAAA-3′ (R) 5′-ACCCGAGTCCCAGACTAGCAGT-3′	261	−11700 (Intron 1a) −11440 (Intron 1b)	Platinum TaqPCRx and Enhancer Solution 1X
5′ Upstream of Exon 1c	(F) 5′-TGGAATCATCTCCCCAGCATTCTCC-3′ (R) 5′-GCCAGGGTATGAGGTCACTGAAGA-3′	450	−6217 (Intron 1b, 430 bp Upstream of Exon 1c) −5768 (Exon 1c)	Taq and Master Amp K
Exon 1c	(F) 5′-TCCTTTCCTCCCTCCTTAGG-3′ (R) 5′-TGGAGCTTCAGGAACTACTGG-3′	476	−5995 (Intron 1b) −5509 (Intron 1c)	Taq and Master Amp K
5′ Upstream of Exon 1d	(F) 5′-GAAAGGTGGAGCTGGGCACG-3′ (R) 5′-CTGGTGAAGCTTTGAGGGAAG-3′	422	−608 (Intron 1c, 425 bp Upstream of Exon 1d) −187 (Exon 1d)	Taq and Master Amp K
Exon 1d	(F) 5′-TGCCAACAGTGCCAAGAGATG-3′ (R) 5′-CATTGGGTGGTCAGGAGAATC-3′	528	−279 (Intron 1c) +249 (Intron 2)	Taq
Exon 2	(F) 5′-ACAGGCCCCAGGTCTATTCTG-3′ (R) 5′-TGCCTATCTCATTGGGTGGTCAG-3′	362	−104 (Intron 1) +258 (Intron 2)	Taq
Exon 3	(F) 5′-GGTGACAGATTGGATGTGCATG-3′ (R) 5′-CTTCCCACAGCTATCATCACTC-3′	326	+2202 (Intron 2) +2527 (Intron 3)	Taq
Exon 4	(F) 5′-ATTTCCTGACCTTGTGATCCGCCT-3′ (R) 5′-GCAGCCAGTAAGGTAGAAAGAGCC-3′	311	+3133 (Intron 3) +3443 (Intron 4)	Taq
Exon 5	(F) 5′-GACCAAATGGAACTTGTTGGGGA-3′ (R) 5′-ATTCCTCCTCCACCTACAGGTA-3′	351	+5678 (Intron 4) +6028 (Exon 6)	Taq
Exon 6	(F) 5′-CAATTGCCCTGGAAGTGCTGAA-3′ (R) 5′-GTGTGCAGAATGTTGAGCACGT-3′	309	+5840 (Exon 5) +6148 (Intron 6)	Taq
Exon 7	(F) 5′-GTGTTGGGATTACAGGTGTGAG-3′ (R) 5′-TTACCTAGTCAGGTTCCCGTG-3′	424	+7468 (Intron 6) +7891 (Intron 7)	Taq
Exon 8	(F) 5′-CTGGACACGTATTCTCATCTG-3′ (R) 5′-TATGTCAGCCTGTCAGTCCACT-3′	539	+8010 (Intron 7) +8548 (Exon 8)	Taq
Exon 8	(F) 5′-AAGCAGGCTGAAAGGCCAGCTT-3′ (R) 5′-CGAGTCTTCCCCAAATGGACTT-3′	469	+8269 (Exon 8) +8737 (Exon 8)	Taq
Exon 8	(F) 5′-AGTGGACTGACAGGCTGACATA-3′ (R) 5′-GATTCTAAGAGGATGTGCTGTGG-3′	501	+8527 (Exon 8) +9027 (Exon 8)	Taq
Exon 8	(F) 5′-CCATGGGTACCTAGAAAGACATC-3′ (R) 5′-AGCCTCAGATTGGGCTAGGGCA-3′	592	+8805 (Exon 8) +9396 (Exon 8)	Taq
Exon 8	(F) 5′-GATGGCCTGTAGCAATGAGGCT-3′ (R) 5′-TGCGCCAATCACTTTTCCTCCT-3′	560	+9240 (Exon 8) +9799 (Downstream of Exon 8)	Taq

F, forward; R, reverse.

Nucleotide positions are counted from A of the start codon on the genomic stretch of the *SRR* gene.

Taq polymerase was purchased from Takara (Tokyo, Japan). Master Amp buffer was from Epicentre (Madison, Wisconsin), and Platinum TaqPCRx/Enhancer Solution were from Invitrogen (Carlsbad, California).

the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were carried out using the Expand Long Template PCR System (Buffer 1; Roche, Indianapolis, Indiana), and the following two gene-specific primers located in exon 3 (Figure 1) and the AP1/AP2 primers supplied by the manufacturer: for the first PCR: 5′-AGCTTCTGACGGCATTGAGAGCACCACG (5′ end at nt +199 on the cDNA sequence; A of the initiation codon was designated as +1), and for the nested PCR: 5′-TTCGCGTTCCT-GCAGATAAGATTTG (5′ end at nt +287 on the cDNA sequence). The genomic structure was predicted by comparing the cDNA sequence with the *Homo sapiens* chromosome 17 genomic contig (accession No. NT_010718). This structure was confirmed by sequencing each exon and flanking intron portion of the genome (see Mutation Screening later in the article).

RNase Protection Assay (RPA) of SRR

Four DNA fragments corresponding to isoforms a, b, c, and d were amplified from Marathon human brain cDNA (BD Biosciences Clontech), using the following primer sets (see Figures 1 and 2): for isoform a, 5′-GGCCAGGCTCTCCCGGAGCT (forward) and 5′-CACATTTGAAGAAAAGATTGCG (named Ex2R, a reverse primer located in exon 2); for isoform b, 5′-GCT-

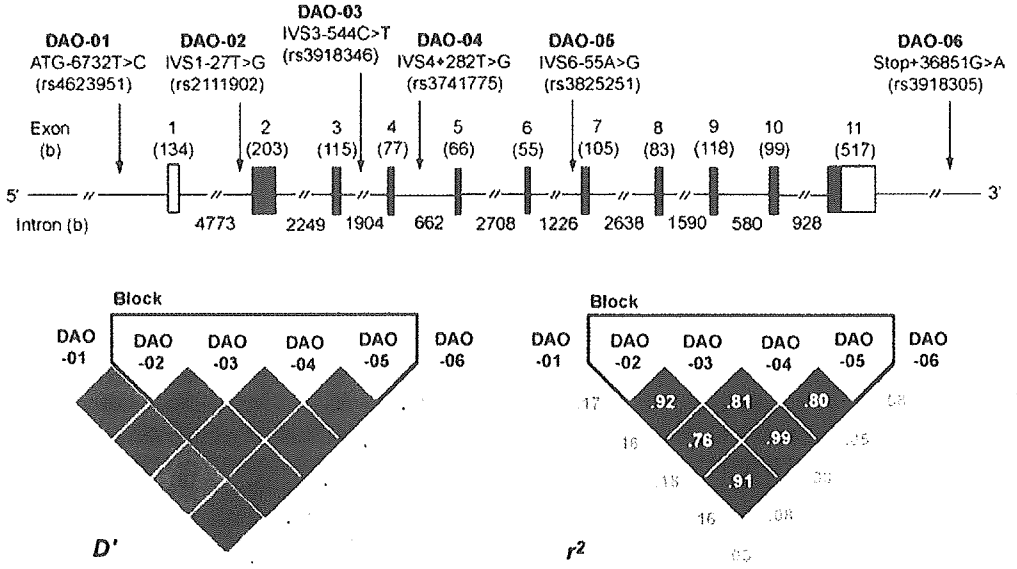

Figure 3. Genomic structure, location of polymorphic sites and haplotype block structure for human *DAO*. In the top panel, exons are denoted by boxes, with untranslated regions in white and translated regions in black. The sizes of exons (b) and introns (b) are also shown. The "rs" number of each single nucleotide polymorphism (SNP) is the National Center for Biotechnology Information SNP cluster ID from the dbSNP database (http://www.ncbi.nlm.nih.gov/SNP/). In the lower panel, the haplotype block structure of *DAO* is shown, with linkage disequilibrium parameters *D'* (left) and r^2 (right). The *D'* and r^2 values were calculated from the sample set B.

GCGCGTGCGCAGAGGTG and Ex2R; for isoform c, 5'-TTCCATATGGAACCAACTCGCCT and Ex2R; for isoform d, 5'-CTCTTCAATAAACATACTGTCTC and Ex2R. Note that these four fragments comprise a common 143 nt sequence and each isoform-specific 60–75 nt sequence. Similarly, a region common to the four isoforms, as well as β-actin, were amplified using the primer sets, 5'-GGCGTCAGAAGCTTGGTTCCT (forward)/5'-TGTCCCTTGTCCAGCTATCACT (reverse), and 5'-GCGGACTATGACTTAGTTGCGT (forward)/5'-TAAAGCCATGCCAATCTCATCTTG (reverse), respectively. These PCR amplicons were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, California), and sequence integrity was confirmed. Antisense [^{32}P]-UTP-labeled riboprobes were synthesized using these constructs and the MAXIscript kit (Ambion, Austin, Texas). An RNase protection assay was performed using Human Brain Total RNA (BD Biosciences Clontech) or yeast total RNA, and the RPAIII kit (Ambion), according to the manufacturer's instructions.

Mutation Screening of SRR

Genomic DNA was isolated from blood samples using standard methods. The complete coding region, exon/intron boundaries, and the 5' upstream regions of *SRR* were screened for polymorphisms by direct sequencing of PCR products from 30 unrelated schizophrenia samples. The primers and DNA polymerases used for amplification are listed in Table 1. The PCR was performed with an initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 15 sec, 55°–70°C (optimized for each primer pair) for 15 sec, 72°C for 45 sec, and a final extension at 72°C for 2 min. Direct sequencing of PCR products was performed using the BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Applied Biosystems, Foster City, California) and the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). Polymorphisms were detected using the SEQUENCHER program (Gene Codes Corporation, Ann Arbor, Michigan).

Selection of Polymorphisms for DAO

For genetic analysis of *DAO*, we first chose three single nucleotide polymorphisms (SNPs) previously reported to be associated with schizophrenia (DAO-02, DAO-03, and DAO-04 in Figure 3; Chumakov et al 2002; Liu et al 2004; Schumacher et al 2004). To examine the 5' and 3'-regions of the gene more thoroughly, we selected SNPs from databases including the Celera Discovery System (Celera; http://www.celeradiscoverysystem.com/) and the Entrez SNP on NCBI (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/). Three additional SNPs—DAO-01, DAO-05, and DAO-06 (Figure 3)—were analyzed after an examination for informativeness using 30 randomly chosen schizophrenic samples (minor allele frequency > .01). We found one missense polymorphism (992G>T, Gly331Val, rs4262766) in the database, but in our screening panel, this polymorphism was always Gly331.

SNP Genotyping

The SNPs were typed in all samples using the TaqMan system (Applied Biosystems). Probes and primers were designed using Assays-by-Design SNP genotyping (Applied Biosystems). The PCR reactions were performed in an ABI 9700 thermocycler and fluorescence was determined using an ABI 7900 sequence detector single point measurement and SDS v2.0 software (Applied Biosystems). Each marker was checked for allele-inheritance inconsistency within a pedigree of the sample set C using PEDCHECK software (O'Connell and Weeks 1998), and no inconsistencies were found, proving the accuracy of the genotyping.

Determination of Total Serine, D-Serine, and L-Serine

Measurement of total, D-, and L-serine levels was carried out according to established methods using a column-switching high-performance liquid chromatography (HPLC) system (Shi-

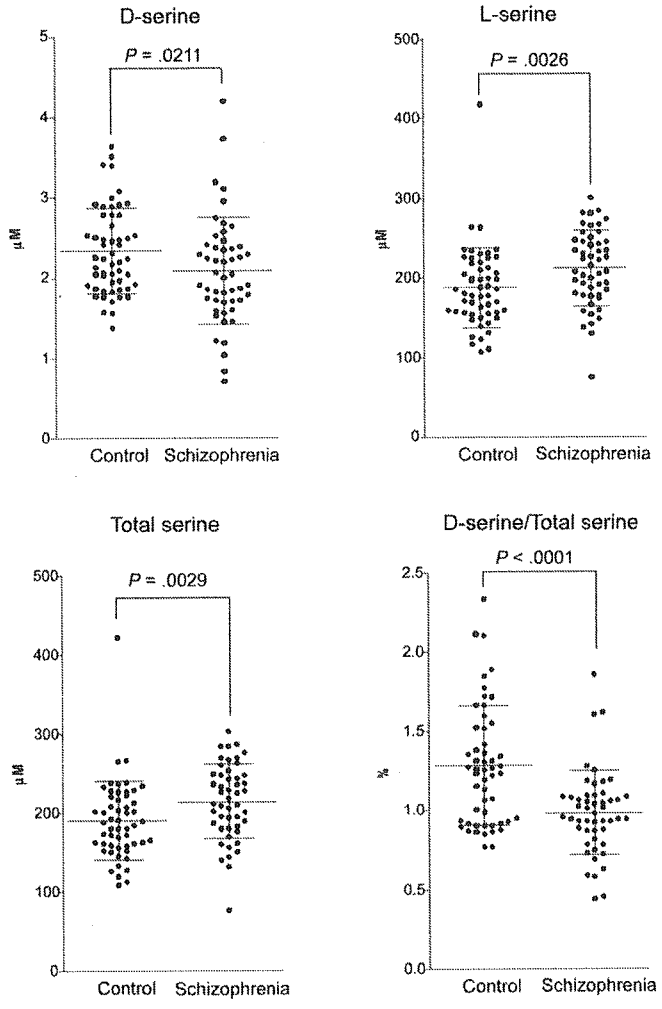

Figure 4. Significant differences in serum levels of serine isomers between healthy control subjects and schizophrenia patients. Examined samples included 52 control subjects and 50 patients with schizophrenia. Horizontal bars show mean ± SD.

madzu Corporation, Kyoto, Japan). A 20-μL aliquot of the human serum was added to 20 μL of .1 M borate buffer (pH 8.0) and 60 μL of 50 mmol/mL 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F; Tokyo Kasei Kogyo, Tokyo, Japan) in CH_3CN. The reaction mixture was then heated at 60°C for 1 min and immediately supplemented with 100 μL of H_2O/CH_3CN (90/10) containing .1% trifluoroacetic acid (TFA) to stop the reaction. A 10-μL aliquot of the resultant solution was injected into the HPLC system. A reversed-phase ODS column (TSKgel ODS-80Ts [Tosoh Corporation, Tokyo, Japan] as Column 1) was used for the separation and quantification of total (D- and L-) serine, and the gradient elution of the mobile phase was maintained at a constant flow rate of .8 mL/min. Mobile phase 1a consisted of H_2O/CH_3CN (90/10) containing .1% TFA, and phases 1b and 1c, of H_2O/CH_3CN (10/90) containing .1% TFA and CH_3CN, respectively. The time program for gradient elution was as follows: 0–25 min 1a : 1b : 1c = 92 : 8 : 0; 25–35 min 1a : 1b : 1c = 0 : 100 : 0; and 35–45 min, 1a : 1b : 1c = 0 : 0 : 100. The chiral column (Column 2) used for the separation and quantification of D- and L-serine with NBD-F comprised two Sumichiral OA-2500 columns (S) (Sumika Chemical Analysis Service, Osaka, Japan), which were connected in tandem. The mobile phase was 15 mmol/mL citric acid in MeOH. The flow rate was isocratically pumped at .8 mL/min. The column temperature of all columns was maintained at 35°C. Fluorescence detection was performed at 530 nm with an excitation wavelength at 470 nm.

Statistical Analyses

Allelic and genotypic frequencies of markers between patients and control subjects in the case–control study were assessed using Fisher's Exact Test. Haplotype frequencies, normalized linkage disequilibrium (LD) coefficient D' and squared correlation coefficient r^2 in the sample set B were calculated using the expectation-maximization algorithm implemented in COCAPHASE software (Dudbridge 2003) (http://www.hgmp.mrc.ac.uk/~fdudbrid/software/). To examine haplotype block structures in the genomic region of *DAO*, we used the Haploview program (Barrett et al 2005; http://www.broad.mit.edu/mpg/haploview/).

All members of the 124 families in sample set C were analyzed using the pedigree disequilibrium test (PDT) program, v3.12 (http://www.chg.duke.edu/software/pdt.html; Martin et al 2000). The complete 80 trio set in sample set C was also analyzed using the extended transmission disequilibrium test (ETDT) algorithm, v2.2 (Sham and Curtis 1995). Empirical significance levels of the ETDT results were simulated from 10,000 Monte Carlo permutations using the MCETDT program, version 1.3 (http://www.mds.qmw.ac.uk/statgen/dcurtis/software.html; Zhao et al 1999). TRANSMIT software (Clayton 1999; http://watson.hgen.pitt.edu/docs/transmit.html) was run as a global test of haplotype transmission for the set of complete 80 trios. Genetic Power Calculator (Purcell et al 2003; http://statgen.iop.kcl.ac.uk/gpc/) was used to compute statistical power.

The differences in serum levels of D-serine, and ratio (%) of D-serine to total (D- and L-) serine between the two groups and among multiple groups were examined using the two-tailed Mann–Whitney U test and the Kruskal–Wallis test.

Results

cDNA and Genomic Structures and Polymorphisms of SRR

The cDNA sequence of the protein coding region for human *SRR* and its genomic structure have been reported (De Miranda et al 2000) and are thought to contain no 5' untranslated exons. Our current 5'-RACE analysis and comparison of cDNA and genomic sequences revealed the existence of four novel 5' untranslated exons, herein referred to as exons 1a–1d, and showed that the alternative use of these 5' end exons gives rise to four different transcripts in the brain; we designate these mRNA variants isoforms a, b, c, and d (Figure 1). These results suggest that at least four promoters, each flanking the 5' portion of exons 1a, 1b, 1c, or 1d, drive and control the expression of *SRR*. We deposited this genomic information into GenBank under the Accession Numbers AY743705, AY743706, AY743707, and AY743708. The reported cDNA sequence (De Miranda et al 2000) started from our exon 1d but contained unknown sequences that could not be found in the human genome sequence database. The University of California at Santa Cruz (UCSC) July 2003 draft assembly of the human genome (http://genome.ucsc.edu/) displays our isoform b structure as a genomic organization of *SRR*. Interestingly, the exon 1d sequences are used as intronic sequences of isoforms a, b, and c (Figure 1). We previously reported this type of rare genomic organization in a different gene (Kikuchi et al 2003a, 2003b; Yoshikawa et al 1998).